CHROM. 16,102

DETERMINATION OF COUMARIN ANTICOAGULANT RODENTICIDE RESIDUES IN ANIMAL TISSUE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

II. FLUORESCENCE DETECTION USING ION-PAIR CHROMATOGRAPHY

K. HUNTER

Department of Agriculture and Fisheries for Scotland, Agricultural Scientific Services, East Craigs, Edinburgh EH12 8NJ (U.K.)

(Received June 26th, 1983)

SUMMARY

A high-performance liquid chromatographic method was developed for the determination of warfarin, coumatetralyl, bromadiolone, difenacoum and brodifacoum in animal tissues using fluorescence detection. Ion-pair chromatography, with the tetrabutylammonium ion as counter-ion, was used to take full advantage of their native fluorescence. Detection limits in liver tissue after gel permeation clean-up were 0.002 mg kg^{-1} for coumatetralyl, difenacoum and bromdifacoum, 0.008 mg kg^{-1} for bromadiolone, and 0.01 mg kg^{-1} for warfarin.

INTRODUCTION

The coumarin-based compounds warfarin [3-(3-oxo-1-phenylbutyl)-4-hydroxycoumarin], coumatetralyl [3-(1,2,3,4-tetrahydro-1-naphthyl)-4-hydroxy-coumarin], difenacoum[3-(3-biphenyl-4-yl-1,2,3,4-tetrahydro-1-naphthyl)-4-hydroxy-coumarin], brodifacoum (3-[3-(4′-bromobiphenyl-4-yl)-1,2,3,4-tetrahydro-1-naptyl)-4-hydroxycoumarin) and bromadiolone (3-[3-(4′-bromobiphenyl-4-yl)-3-hydroxy-1-phenylpropyl]-hydroxycoumarin) have all been used as anticoagulant rodenticides. Their use had led to a requirement for an analytical method for this family of compounds in cases of suspected poisoning in non-target species. Gas chromatographic (GC) methods have been described for the determination of warfarin[1,2], but GC methods have not proved reliable for difenacoum and brodifacoum because of either thermal degradation of the parent compound in the chromatographic column or incomplete derivatisation in the preparation of silyl and methyl adducts[3,4]. High-performance liquid chromatography (HPLC) has been used to determine warfarin[5], coumatetralyl[6], brodifacoum[7] and difenacoum[6] residues in animal tissues and has afforded the possibility of multi-residue analysis for these rodenticides[4,8].

The native fluorimetric properties of warfarin have been described by Corn and Berberich[9], and other coumarin rodenticides have similar characteristics. Flu-

orescence detection combined with HPLC separation might make the sensitive multi-residue analysis of these compounds possible, but in most reports full exploitation of their native fluorescence has been precluded because of the use of acidic mobile phases which quench the fluorescence. Although Mundy and Machin[4] successfully employed exclusion HPLC to make use of the full fluorimetric potential of warfarin, coumatetralyl, difenacoum and brodifacoum, the latter three compounds were unresolved. HPLC post-column pH-switching techniques have permitted the sensitive fluorimetric detection of coumarin rodenticides. The use of this technique was described for the normal-phase determination of warfarin and its metabolites[10] and has been reported recently by the author for both normal and reversed-phase systems for the multi-residue analysis of coumarin rodenticides, including bromadiolone, in animal tissues[11].

Acidic mobile phases have been used for reversed-phase HPLC of coumarin rodenticides because these compounds act as weak acids and at neutral pH some are not retained by reversed-phase columns while others elute as broad ill-defined peaks, hence the use of ion-suppression techniques has been necessary. An alternative HPLC strategy for this type of compound is possible by using ion-pairing techniques. This chromatographic mode has been used for determining warfarin in rodenticide concentrates[12]. Ion-pair chromatography of acidic compounds can be achieved using a cationic counter-ion with the mobile phase buffered at pH 7.5, and such conditions should be ideally suited to exploit the native fluorescence of coumarin rodenticides. This report describes the development of an ion-pairing HPLC method for analysing coumarin anticoagulant rodenticides in animal tissues using fluorescence detection and compares it with the post-column pH-switching techniques described previously[11].

EXPERIMENTAL

Materials and apparatus

HPLC grade solvents were supplied by Rathburn, Walkerburn, U.K. Low UV PIC A reagent, a commercially prepared tetrabutylammonium phosphate ion-pairing reagent buffered at pH 7.5, was purchased from Waters Assoc., Hartford, Northwich, U.K. All other chemicals were supplied by BDH, Poole, U.K., and were of AnalaR grade. Bio-Beads SX-3 (200–400 mesh) were obtained from Bio-Rab Labs., Watford, U.K. Reference standards of warfarin and coumatetralyl were obtained from the Laboratory of the Government Chemist, London. Difenacoum and brodifacoum were supplied by Sorex (London), Wembley, U.K., and bromadiolone by Rentokil, Kirkby, U.K.

Tissue samples were homogenised with an Ultra-Turrax 18N tissue disperser. The gel permeation chromatographic clean-up system using Bio-Beads SX-3 in hexane–chloroform–acetone (75:20:5) has been described elsewhere[11]. The HPLC system was made up of a Spectra-Physics SP 8700 solvent delivery unit with a Rheodyne 7125 injector (20 μl), and a Perkin-Elmer LS-4 fluorescence detector. The column (250 × 4.6 mm I.D.) was slurry packed, by a Haskel pneumatic amplifier pump, with ODS-Hypersil (5 μm) using isopropanol as the slurry medium and methanol as the packing medium.

Extraction and clean-up

Rodenticide residues were extracted from animal tissues by maceration in chloroform–acetone (1:1) and the resulting extracts cleaned-up by gel permeation chromatography on Bio-Beads SX-3 using hexane–chloroform–acetone (75:20:5) as the eluent[11]. Cleaned-up extracts were carefully evaporated to dryness and redissolved in the HPLC mobile phase (1 ml). Solutions of reference standards were also prepared in the HPLC mobile phase.

HPLC analysis

Solutions containing PIC A reagent were prepared in methanol and in water according to the manufacturer's instructions. It was necessary to filter methanol solutions to remove precipitated buffer salts. Prior to and during use both solutions were degassed by helium sparging. Several mixtures of these two solutions, containing 55–80% methanol, were used as mobile phases at a flow-rate of 1.5 ml min⁻¹ to determine the elution characteristics of the coumarin rodenticides from an ODS-Hypersil column. After equilibration of the chromatographic system with each mobile phase, 20-μl aliquots of extracts or reference standards were injected. The fluorometric response at an excitation wavelength of 310 nm and an emission wavelength of 390 nm was monitored. Both isocratic elution and programmed gradient elution were evaluated.

RESULTS AND DISCUSSION

Extraction and clean-up

An earlier extraction study[11] had shown that residues of coumarin anticoagulant rodenticides in animal tissues could be extracted efficiently with a chloroform–acetone (1:1) mixture. Recoveries of warfarin, coumatetralyl, bromadiolone, difenacoum and brodifacoum from spiked liver tissue were ≥90% using this mixture. In the previous study a clean-up method employing gel permeation chromatography permitted effective HPLC determination of coumarin rodenticides at levels down to 0.002–0.01 mg kg⁻¹ using fluorescence detection. This gel permeation clean-up also proved adequate for the present study.

Fluorescence spectral characteristics

The fluorimetric spectral characteristics of each rodenticide were examined using solutions of each compound in the mobile phase solvents. The excitation and emission spectra were essentially identical to those reported earlier[11]; all five rodenticides exhibited maximum emission at 390 mm when excited at 310 nm but for bromadiolone, difenacoum and brodifacoum the intensity of the emission at 390 nm was approximately doubled by excitation at 255 nm.

Ion-pair chromatography

Preliminary experiments using methanol and phosphate buffer pH 7.5 showed that the tetrabutylammonium ion (TBA⁺) could be used as a counter-ion for the ion-pair chromatography of coumarin rodenticides. Mobile phases with a pH in the range 6.5–8.5 were satisfactory for chromatographic purposes purposes and were suitable for fluorimetric detection. Experiments were done with various concentra-
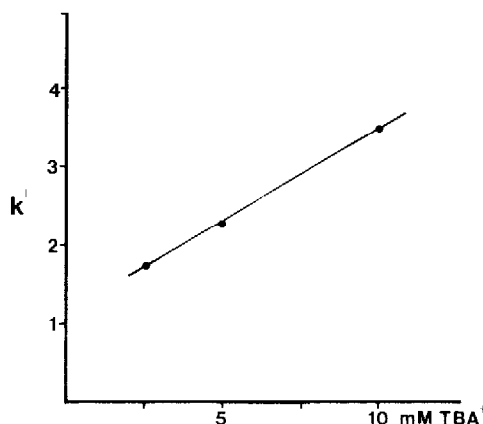
Fig. 1. Influence of counter-ion concentration on the capacity factor (k') of difenacoum. Column, ODS-Hypersil (250 × 4.6 mm I.D.); mobile phase, methanol–0.025 M phosphate buffer pH 7.5 (76.5:23.5); counter-ion, tetrabutylammonium hydroxide (40% solution) added to mobile phase to give the concentrations indicated and the pH adjusted to 7.5 with hydrochloric acid; flow-rate, 1.5 ml min⁻¹; injections, 200 ng of difenacoum in 20 μl of mobile phase.

tions of TBA$^+$ in the mobile phase. As the concentration of the counter-ion was increased from 0.0025 to 0.01 M there was a linear increase in the retention of all five rodenticides. This effect of counter-ion concentration on the capacity factor (k') of difenacoum is shown in Fig. 1.

Subsequently the mobile phase modifier, PIC A reagent, was employed to make up mobile phases. The final concentration of TBA$^+$ in the mobile phase was 0.005 M using this reagent. The elution characteristics of the coumarin rodenticides were determined by varying the proportion of methanol in the mobile phase for isocratic chromatographic examination. The order of elution was the same as that observed using ion-suppression reversed-phase chromatography[11], warfarin was eluted first followed by coumatetralyl, bromadiolone, difenacoum and finally brodifacoum. Difenacoum and brodifacoum each eluted as a single peak with no resolution of their *cis*- and *trans*-isomers. The influence of methanol concentration in the mobile phase

TABLE I

INFLUENCE OF METHANOL CONCENTRATION IN THE MOBILE PHASE ON THE CAPACITY FACTORS OF COUMARIN RODENTICIDES

Column, ODS-Hypersil (250 × 4.6 mm I.D.); mobile phase, methanol–water containing PIC A reagent, 1.5 ml min⁻¹.

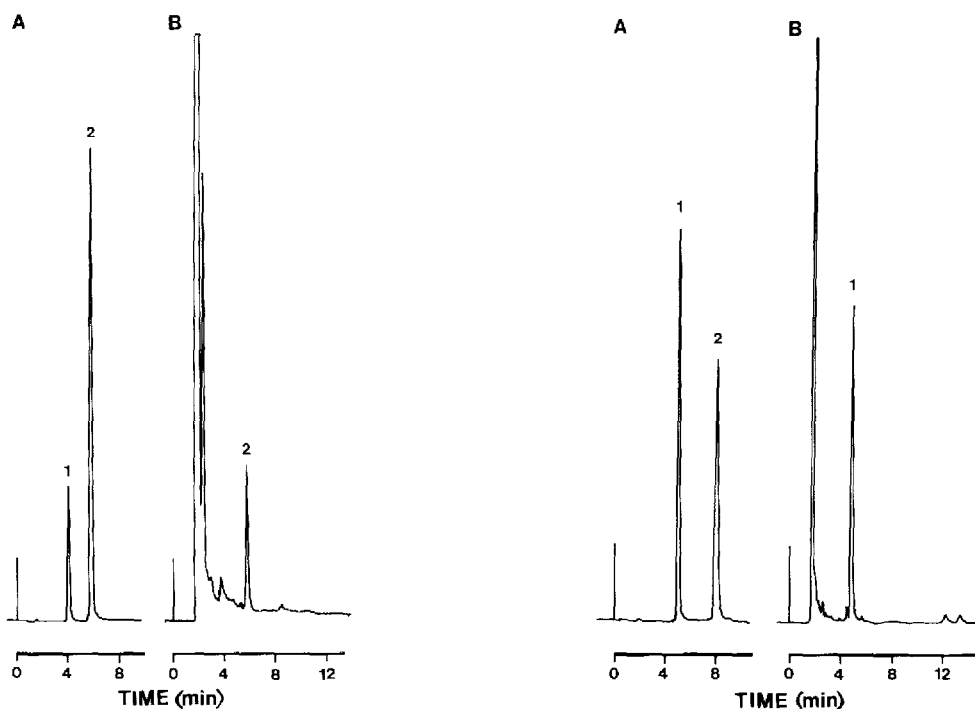|	Percentage methanol in mobile phase						
	80	*78*	*75*	*72*	*65*	*58*	*55*
Warfarin	—	—	—	—	—	1.33	1.89
Coumatetralyl	—	—	0.28	—	1.0	2.44	8.56
Bromadiolone	—	—	1.89	2.89	—	—	—
Difenacoum	1.11	1.67	2.83	4.44	—	—	—
Brodifacoum	2.11	3.33	5.67	—	—	—	—

Fig. 2. Ion-pair chromatography of warfarin (1) and coumatetralyl (2) on ODS-Hypersil. Mobile phase, methanol–water (58:42) containing PIC A reagent; flow-rate, 1.5 ml min^{-1}; fluorescence detection, (A) × 1, (B) × 6; λ_{ex} = 310 nm, λ_{em} = 390 nm. (A) Reference standards of warfarin (40 ng) and coumatetralyl (22.5 ng). (B) Feline liver extract spiked with coumatetralyl (1 ng) ($\equiv$ 0.01 mg kg^{-1}).

Fig. 3. Ion-pair chromatography of difenacoum (1) and brodifacoum (2) on ODS-Hypersil. Mobile phase, methanol–water (78:22) containing PIC A reagent; flow-rate, 1.5 ml min^{-1}; fluorescence detection, × 1; λ_{ex} = 310 nm, λ_{em} = 390 nm. (A) Reference standards of difenacoum (19 ng) and brodifacoum (20 ng). (B) Extract from canine liver containing difenacoum ($\equiv$ 0.58 mg kg^{-1}).

on the k' value of each rodenticide is shown in Table I. A linear relationship between methanol concentration and log k' was observed for coumatetralyl, difenacoum, and brodifacoum but insufficient data were obtained to demonstrate any effect for warfarin and bromadiolone. Because of the large differences in k' a simple isocratic analysis of all five rodenticides was not possible. At least two different isocratic separations were required for convenient multi-residue screening of animal tissue extracts. The separation of warfarin and coumatetralyl and the identification of coumatetralyl in a feline liver extract are shown in Fig. 2. Chromatograms demonstrating the separation of difenacoum and brodifacoum and the determination of a difenacoum residue in a canine kidney extract are shown in Fig. 3.

The minimum detectable amounts for coumatetralyl, difenacoum and brodifacoum were 15 pg, for bromadiolone 70 pg and for warfarin 100 pg. The fluorimetric responses of the rodenticides were linear up to at least 2 μg. For animal tissue extracts detection limits using gel permeation clean-up were 0.002 mg kg^{-1} (wet weight) for coumatetralyl, difenacoum and brodifacoum, 0.008 mg kg^{-1} for bromadiolone and 0.01 mg kg^{-1} for warfarin. Excitation of bromadiolone, brodifacoum and difenacoum
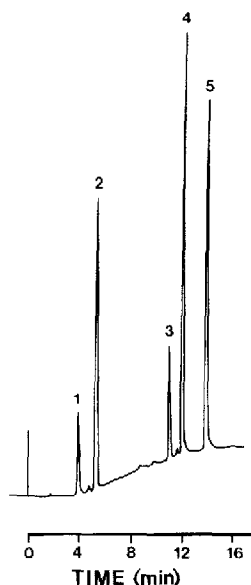
Fig. 4. Ion-pair gradient separation of coumarin rodenticides on ODS-Hypersil. Mobile phase, methanol–water containing PIC A reagent; gradient, 58% methanol to 78% over 8 min, then to 88% methanol over the following 6 min with a 4-min hold at the final conditions; flow-rate, 1.5 ml min^{-1}; fluorescence detection, × 2; λ_{ex} = 310 nm, λ_{em} = 390 nm. Reference standards: 1 = warfarin 8 ng; 2 = coumatetralyl 4.5 ng; 3 = bromadiolone 9.5 ng; 4 = difenacoum 7.5 ng; 5 = brodifacoum 8 ng.

at 255 nm increased the response but did not change the signal-to-noise ratio, therefore excitation at 310 nm was adopted for routine use.

Experiments were done to evaluate programmed gradient elution for multi-residue analysis. Although it was possible to achieve a satisfactory separation of the five rodenticides, excessive baseline drift at high sensitivities was a limiting factor in the application of gradient elution for the determination of low residues. The gradient separation of the coumarin rodenticides is illustrated in Fig. 4.

Comparisons of this ion-pair chromatographic method with the two pH-switching techniques described earlier[11] made it clear that no simple isocratic analysis of all the coumarin rodenticides examined was possible in any of the three chromatographic modes. Gradient elution was possible in the ion-pair method and in the reversed-phase pH-switching technique. However in both cases ultimate sensitivity was restricted because of baseline drift during gradient elution, and this problem was more acute with the ion-pairing system. Overall the detection limits using ion-pair chromatography and the reversed-phase pH-switching technique were similar, and were slightly better than those achieved using the normal-phase pH-switching technique. The ion-pair method had advantages in that few difficulties were observed from interfering co-extractives in cleaned-up animal tissue extracts and no additional reagent pump and reaction coil were necessary. The principal advantage of the pH-switching techniques over ion-pair chromatography was that some measure of confirmation of the identity of rodenticide residues was possible by re-chromatographing extracts in the absence of the post-column reagents.

CONCLUSIONS

    HPLC determination by ion-pair chromatography provided a means of fully utilising the native fluorescence of coumarin based anticoagulant rodenticides. Residues of warfarin, coumatetralyl, bromadiolone, difenacoum and brodifacoum could be measured at very low levels in animal tissue extracts with little or no interference from co-extracted material. The method provided an alternative and complementary fluorometric assay to that achieved by post-column pH switching techniques.

ACKNOWLEDGEMENTS

REFERENCES

1 E. M. Odam and M. G. Townsend, *Analyst (London)*, 101 (1976) 478.
2 D. G. Kaiser and R. S. Martin, *J. Pharm. Sci.*, 63 (1974) 1579.
3 S. H. Yuen, *Analyst (London)*, 103 (1978) 842.
4 D. E. Mundy and A. F. Machin, *J. Chromatogr.*, 234 (1982) 427.
5 D. E. Mundy, M. P. Quick and A. F. Machin, *J. Chromatogr.*, 121 (1976) 335.
6 D. E. Mundy and A. F. Machin, *J. Chromatogr.*, 139 (1977) 321.
7 K. G. Koubek, J. P. Ussary and R. E. Haulsee, *J. Ass. Offic. Anal. Chem.*, 62 (1979) 1297.
8 A. J. Kieboom and C. G. Rammell, *Bull. Environ. Contam. Toxicol.*, 26 (1981) 674.
9 M. Corn and R. Berberich, *Clin. Chem.*, 13 (1967) 126.
10 S. H. Lee, L. R. Field, W. N. Howald and W. F. Trager, *Anal. Chem.*, 53 (1981) 467.
11 K. Hunter, *J. Chromatogr.*, 270 (1983) 267.
12 W. A. Trujillo, *J. Liquid Chromatogr.*, 3 (1980) 1219.